

## Example 125

Inhibition of Cellular Viability in Tumor Cell Lines  
Treated with Mitotic Kinesin Inhibitors**[1246]** Materials and Solutions:**[1247]** Cells: SKOV3, Ovarian Cancer (human).**[1248]** Media: Phenol Red Free RPMI+5% Fetal Bovine Serum+2mM L-glutamine.**[1249]** Colorimetric Agent for Determining Cell Viability: Promega MTS tetrazolium compound.**[1250]** Control Compound for max cell kill: Topotecan,**[1251]** Procedure: Day 1—Cell Plating:**[1252]** Adherent SKOV3 cells are washed with 10 mLs of PBS followed by the addition of 2mLs of 0.25% trypsin and incubation for 5 minutes at 37° C. The cells are rinsed from the flask using 8 mL of media (phenol red-free RPMI+ 5% FBS) and transferred to fresh flask. Cell concentration is determined using a Coulter counter and the appropriate volume of cells to achieve 1000 cells/1004 is calculated. 100  $\mu$ L of media cell suspension (adjusted to 1000 cells/100  $\mu$ L) is added to all wells of 96-well plates, followed by incubation for 18 to 24 hours at 37° C., 100% humidity, and 5% CO<sub>2</sub>, allowing the cells to adhere to the plates.**[1253]** Procedure: Day 2—Compound Addition:**[1254]** To one column of the wells of an autoclaved assay block are added an initial 2.5  $\mu$ L of test compound(S) at 400 $\times$  the highest desired concentration. 1.25  $\mu$ L of 400 $\times$  (400  $\mu$ M) Topotecan is added to other wells (ODs from these wells are used to subtract out for background absorbance of dead cells and vehicle). 500  $\mu$ L of media without DMSO are added to the wells containing test compound, and 250  $\mu$ L to the Topotecan wells. 250  $\mu$ L of media+0.5% DMSO is added to all remaining wells, into which the test compound(S) are serially diluted. By row, compound-containing media is replica plated (in duplicate) from the assay block to the corresponding cell plates. The cell plates are incubated for 72 hours at 37° C., 100% humidity, and 5% CO<sub>2</sub>.**[1255]** Procedure: Day 4—MTS Addition and OD Reading:**[1256]** The plates are removed from the incubator and 40  $\mu$ L MTS/PMS is added to each well. Plates are then incubated for 120 minutes at 37° C., 100% humidity, 5% CO<sub>2</sub>, followed by reading the ODs at 490 nm after a 5 second shaking cycle in a ninety-six well spectrophotometer.

## Data Analysis

**[1257]** The normalized % of control (absorbance-background) is calculated and an XLfit is used to generate a dose-response curve from which the concentration of compound required to inhibit viability by 50% is determined. The compounds of the present invention show activity when tested by this method.

## Example 126

## Application of a Mitotic Kinesin Inhibitor

**[1258]** Human tumor cells Skov-3 (ovarian) were plated in 96-well plates at densities of 4,000 cells per well, allowed to adhere for 24 hours, and treated with various concentrations of the test compounds for 24 hours. Cells were fixed in 4% formaldehyde and stained with antitubulin antibodies (subsequently recognized using fluorescently-labeled secondary antibody) and Hoechst dye (which stains DNA).**[1259]** Visual inspection revealed that the compounds caused cell cycle arrest.

## Example 127

Inhibition of Cellular Proliferation in Tumor Cell  
Lines Treated with Mitotic Kinesin Inhibitors**[1260]** Cells were plated in 96-well plates at densities from 1000-2500 cells/well of a 96-well plate and allowed to adhere/grow for 24 hours. They were then treated with various concentrations of drug for 48 hours. The time at which compounds are added is considered T<sub>0</sub>. A tetrazolium-based assay using the reagent 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (I.S> U.S. Pat. No. 5,185,450) (see Promega product catalog #G3580, CellTiter 96® AQ<sub>ueous</sub> One Solution Cell Proliferation Assay) was used to determine the number of viable cells at T<sub>0</sub> and the number of cells remaining after 48 hours compound exposure. The number of cells remaining after 48 hours was compared to the number of viable cells at the time of drug addition, allowing for calculation of growth inhibition.**[1261]** The growth over 48 hours of cells in control wells that had been treated with vehicle only (0.25% DMSO) is considered 100% growth and the growth of cells in wells with compounds is compared to this. Mitotic kinesin inhibitors inhibited cell proliferation in human ovarian tumor cell lines (SKOV-3).**[1262]** A Gi<sub>50</sub> was calculated by plotting the concentration of compound in  $\mu$ M vs the percentage of cell growth of cell growth in treated wells. The Gi<sub>50</sub> calculated for the compounds is the estimated concentration at which growth is inhibited by 50% compared control, i.e., the concentration at which:

$$100 \times [(Treated_{48} - T_0) / (Control_{48} - T_0)] = 50.$$

**[1263]** All concentrations of compounds are tested in duplicate and controls are averaged over 12 wells. A very similar 96-well plate layout and Gi<sub>50</sub> calculation scheme is used by the National Cancer Institute (see Monks, et al., J. Natl. Cancer Inst. 83:757-766 (1991)). However, the method by which the National Cancer Institute quantitates cell number does not use MTS, but instead employs alternative methods.

## Example 128

**[1264]** Calculation of IC<sub>50</sub>**[1265]** Measurement of a composition's IC<sub>50</sub> uses an ATPase assay. The following solutions are used: Solution 1 consists of 3 mM phosphoenolpyruvate potassium salt (Sigma P-7127), 2 mM ATP (Sigma A-3377), 1 mM IDTT (Sigma D-9779), 5  $\mu$ M paclitaxel (Sigma T-7402), 10 ppm, antifoam 289 (Sigma A-8436), 25 mM Pipes/KOH pH 6.8 (Sigma P6757), 2 mM MgCl<sub>2</sub> (VWR JT400301), and 1 mM EGTA (Sigma E3889). Solution 2 consists of 1 mM NADH (Sigma N8129), 0.2 mg/ml BSA (Sigma A7906), pyruvate kinase 7 U/ml, L-lactate dehydrogenase 10 U/ml (Sigma P0294), 100 nM motor domain of a mitotic kinesin, 50  $\mu$ g/ml microtubules, 1 mM DTT (Sigma D9779), 5  $\mu$ M paclitaxel (Sigma T-7402), 10 ppm antifoam 289 (Sigma A-8436), 25 mM Pipes/KOH pH 6.8 (Sigma P6757), 2 mM MgCl<sub>2</sub> (VWR JT4003-01), and 1 mM EGTA (Sigma E3889). Serial dilutions (8-12 two-fold dilutions) of the composition are made in a 96-well microtiter plate (Corning Costar 3695) using Solution 1. Following serial dilution each well has 50  $\mu$ L of Solu-